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Mixed Genotypes Infection With Hepatitis D Virus

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Heterogeneity of hepatitis C viral (HCV) genomes results in escape from immune clearance. Super-infection or mixed infection of different genotypes of HCV are seen commonly in humans. Hepatitis D virus (HDV) is classified into 3 genotypes. This study was planned to investigate if mixed genotypes infection of HDV occurs in humans. HDV genotyping based on restriction fragment length polymorphism (RFLP) was used to screen 60–99 HDV clones from each case of 7 prostitutes and 11 patients. Mixed infections were diagnosed by the finding of two or more different RFLP patterns in a case and were confirmed by sequencing. Five prostitutes had mixed infections of genotypes IIa and IIb HDV, while only 2 patients had mixed infections of genotypes I and II HDV ($P < 0.05$). The heterogeneity in nucleotide sequence was generally below 2% among HDV quasi-species from the same subject, while the heterogeneity was 27.7% between genotypes I and II HDV, and 22.8% between genotypes IIa and IIb HDV from a subject with mixed infection. Multiple HDV clones from the spouses of the 2 index cases were also analyzed. One spouse had mixed infection and the other did not, corresponding to the index cases. In cases with mixed genotypes infections, the prevalence of the minor strain was less than 10% of the total colony population analyzed. ***J. Med. Virol. 57:64–67, 1999.***

KEY WORDS: Genotype; restriction fragment length polymorphism; superinfection, quasi-species; heterogeneity

INTRODUCTION

Heterogeneity of hepatitis C viral (HCV) genomes results in escape from immune clearance, superinfection or mixed infection of HCV and has been reported by many investigators [Farci et al., 1992; Okamoto et al., 1992; Kao et al., 1993; Kao et al., 1994]. In the case of hepatitis D virus (HDV) infection, there is a report of a 1.43% micro-heterogeneity in nucleotide sequences of different clones of HDV from a single subject [Wu et al., 1995b]. HDV is currently classified into three genotypes based on nucleotide sequence comparison [Casey et al., 1993]. The divergence in nucleotide sequences ranges from 5% to 14% among different isolates of the same genotype and from 23% to 34% among different genotypes [Casey et al., 1993; Wu et al., 1995b]. At least two genotypes of HDV have been found in Taiwan [Wu et al., 1995b and 1995c]. Recently, a novel HDV strain with a unique *Xho*I-cleaved restriction fragment length polymorphism (RFLP) of PCR products was isolated from some HDV-infected patients and prostitutes in Taiwan [Wu et al., 1998]. The homologies in whole HDV genomic sequences between the novel strain and genotypes I, II, and III were 72.3%, 77.2%, and 63.0%, respectively. The novel isolates formed a monophyletic group in phylogenetic analysis, with a closer relationship to genotype II. Thus, it is defined as genotype IIb (Accession number in Genbank: AF018077) [Wu et al., 1998].

The most common transmission route of HDV in Taiwan is by sexual contact with prostitutes [Wu et al., 1990]. The high-risk groups such as prostitutes and their sexual contacts thus carry a risk of multiple or re-exposure to this virus. It has been reported that immunization with a recombinant hepatitis delta antigen fails to protect carrier woodchucks from HDV superinfection [Karayiannis et al., 1990]. Re-infection or mixed infection of HDV is highly likely to occur in subjects with risk behaviors. In this study, multiple HDV clones of each patient or prostitute were analyzed by restriction fragment length polymorphism (RFLP) to determine genotypes. The HDV clones from a single subject with more than one RFLP pattern were further sequenced to verify the existence of infection of mixed genotypes.

MATERIALS AND METHODS

Seven HDV-infected prostitutes and 11 patients who had been tested positive for serum HDV RNA were

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included [Wu et al., 1990, 1995b and 1995c]. They were not related to each other. Two HDV-infected spouses of the index cases were also included. They were all positive for serum hepatitis B surface antigen and antibody to HDV antigen (anti-HDV), and they were all negative for immunoglobulin M antibody to hepatitis B core antigen (Ausria II-125, CORAB-M and anti-Delta; Abbott Laboratories, North Chicago, IL). The difference in the prevalence rate of mixed infections of HDV between prostitutes and patients was evaluated by Fisher's exact test.

Reverse transcription polymerase chain reaction (RT-PCR) using primers #120 (homologous to a sequence from nt 889 to nt 912) and #214 (complementary to a sequence from nt 1334 to nt 1313) was performed as reported previously [Chao et al., 1991; Wu et al., 1995b]. In brief, viral RNA was extracted from 50 μ1 of serum, dissolved in 10 μ1 DEPC treated H2O, and heated at 70°C for 10 min. Then cDNA was generated in the presence of reverse transcriptase (SUPERSCRIPT™ II, GIBCOBRL, Life Technologies, Rockville, MD) according to the manufacture's instructions. Each 100 μ1 of PCR reaction mixture contained 5 μ1 of cDNA, 0.5 μ1 (5 units/μ1) of thermostable polymerase (TaKaRa™ Taq™, TAKARA SHUZO CO., LTD., Biomedical group, Shiga, Japan), 10 μ1 of 10 X PCR buffer, 8 μ1 of dNTP mixture (2.5 mM each), 4 μ1 of primers (10 pmol/μ1 each) and 72.5 μ1 of H2O. The PCR was performed in a thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT). It was started with 95°C for 2 min, followed by 35 cycles (each cycle: 95°C for 20 sec, 55°C for 40 sec, 72°C for 1 min) of amplification and ended at 72°C for 10 min. The RT-PCR products were analyzed in 4% agarose gel, followed by ethidium bromide staining. Strict procedures were followed to avoid false positive results [Kwok and Higuchi, 1989]. To avoid contamination, only one serum sample from each of the studied cases and a negative control serum were analyzed in each RT-PCR run. To avoid carry over, all tips used in experiments had filter and were disposed in every step of the experiments. All the buffers, reagents, and primers were separated into aliquots that were discarded after each experiment. The amplified PCR products were ligated into the plasmid pCR2 vector (Original TA Cloning® Kit, Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. The ligation mixture was used to transform the competent *Escherichia coli* strain DH5 α (Gibco BRL, Life Technologies, Gaithersburg, MD) [Chung and Miller, 1988; Wu et al., 1995b]. Sixty-99 colonies from each case were randomly selected. The colonies were lysed by heating at 95°C for 5 minutes, followed by PCR using primers #214 and #120 [Chao et al., 1991; Wu et al., 1995b]. The PCR product generated from each colony was digested with XhoI and analyzed in a 4% agarose gel. Colonies that showed different RFLP patterns were sequenced to confirm the presence of mixed infections. One or more positive colonies were picked up and cultured in Lauria-Bartani medium. Plasmid DNA was extracted and subjected to the dye terminator cycle sequencing reaction according to the standard protocol provided by the manufacturer (Dye terminator cycle sequencing core kit #402117, Perkin Elmer Cetus Corp., Norwalk, CT). The sequencing products were precipitated with alcohol and analyzed in an ABI 373A sequencer (Perkin Elmer Cetus Corp., Norwalk, CT). HDV sequences were aligned by multiple alignments using the Clustal V Program [Higgins et al, 1992]. Phylogenetic analysis using parsimony (PAUP) (Version 3.1.1.; Swofford, 1993) was carried out as described previously [Swofford et al., 1996; Wu et al., 1998].

RESULTS

The expected sizes of PCR products are 446-bp (from nt 889 to nt 1334) and 443-bp for genotypes I and II, respectively [Wu et al., 1995c]. HDV genotypes were screened by XhoI-cleaved RFLP of PCR products. The 446-bp genotype I HDV PCR products were cleaved into a 387-bp fragment and a 59-bp fragment. The 443-bp genotype II HDV PCR products were cleaved into a 303-bp fragment, an 84-bp fragment and a 59-bp fragment [Wu et al., 1995c]. The genotype III HDV does not contain an XhoI cutting site within the amplified sequence. The genotype IIb HDV showed a unique RFLP pattern composed of a 362-bp fragment and an 83-bp fragment, different from those of the 3 genotypes [Wu et al., 1998]. Of the PCR products from the 7 prostitutes, 5 showed the genotype IIa pattern and the remaining 2 showed the genotype IIb pattern. Of the PCR products from the 11 patients, 4 showed the genotype I pattern, 5 showed the genotype IIa pattern and the remaining 2 showed the genotype IIb pattern.

The PCR products from each case were cloned, and 60–99 colonies from each case were analyzed for genotyping. Mixed genotypes infections of HDV were considered if those colonies showed more than one RFLP pattern. Colonies that showed different RFLP patterns were sequenced to confirm the presence of mixed infections. As shown in Table I, there was no significant difference in the number of clones analyzed for each case of the two groups; yet, the prevalence rate of mixed genotypes infection of HDV was significantly higher in prostitutes than in patients (5/7 vs. 2/11, $P < 0.05$). Two of the 11 patients had mixed HDV infections of dominant genotype I and minor genotype IIa. Of the remaining 9 patients without mixed infection, 2 had purely genotype I HDV infection, 5 had only genotype IIa HDV infection and 2 had only genotype IIb HDV infection. Five prostitutes had mixed infections of HDV: 3 had dominant genotype IIa and minor genotype IIb HDV; 2 had genotype IIb as the dominant strain and genotype IIa as the minor strain. Of the remaining 2 prostitutes without mixed infection, both were infected with only genotype IIa HDV. Ten clones of the dominant strain and all the clones of the minor strain from each case with mixed genotypes infection were sequenced. The microheterogeneity of each subgroup (dominant or minor) is generally below 2%, consistent with a previous report [Wu et al., 1995b]. While

TABLE I. Demographic Data and Prevalence of Mixed Genotypes Infections of Hepatitis D Virus in Patients and Prostitutes

	Prostitutes	Patients
No. of cases	7	11
No. of female	7	1
Age, yrs (range)	21–37	23–69
No. of cases with paid-sex	7	8
No. of cases with drug abuse	0	1
Times of paid-sex (range)*	840–28080	0–768
No. of clones/each case	60–96	66–99
No. with mixed infections	5 (71)	2 (18)
Genotype I/IIa[a]	0 (0)	2 (18)
Genotype IIa/IIb[a]	3 (43)	0 (0)
Genotype IIb/IIa[a]	2 (29)	0 (0)
Genotype I only	0 (0)	2 (18)
Genotype IIa only	2 (29)	5 (45)
Genotype IIb only	0 (0)	2 (18)
Genotype III	0 (0)	0 (0)

Estimated total times of paid-sex in the past before inquiry.
[a]The Roman figure indicates genotype, while the front number indicates the dominant strain and the following number indicates the minor strain.
The numbers in parentheses indicate percentage.

TABLE II. Percentage of The Dominant and The Minor Hepatitis D Virus Genotypes in Mixed Infections

			No. (%) of clones with		
No.	Age	Sex	Type I	Type IIa	Type IIb
1	67	F	95 (99)	1 (1)	0
2	50	M	96 (97)	3 (3)	0
P1	31	F	0	95 (99)	1 (1)
P2	23	F	0	4 (5)	75 (95)
P3	21	F	0	8 (10)	76 (90)
P4	34	F	0	56 (93)	4 (7)
P5	33	F	0	82 (95)	4 (5)

P: prostitute; M: male, F: female.

the heterogeneity was 27.7% between genotypes I and II HDV, and 22.8% between genotypes IIa and IIb HDV from a subject with mixed genotypes infection. In cases with mixed genotypes infections, the minor strains ranged from 1% to 10% of the total number of colonies analyzed (Table II).

Multiple HDV clones from the spouses of the 2 index cases were also analyzed. The spouse of the index case who had mixed infections of a dominant genotype I and a minor genotype II had similar composition of mixed genotypes. The spouse of the other index case who had only genotype IIa infection was also infected by HDV of the same genotype.

DISCUSSION

By using type-specific primers in PCR, mixed infections of HCV are found frequently found [Farci et al., 1992; Okamoto et al., 1992; Kao et al., 1993 and 1994]. However, there has been no report of mixed genotypes infections of HDV. Previous studies of HDV genotyping were based on direct sequencing of RT-PCR products, sequencing of a few clones, or RFLP analysis of RT-PCR products of the HDV genomes from patients. These methods are not able to detect minor strains less than 10% of the total viral population [Casey et al., 1993 and 1996; Wu et al., 1995b and 1995c; Lee et al, 1992 and 1996; Niro et al., 1997; Imazeki et al., 1990] In this study, mass screening of HDV genotypes based on RFLP analysis of multiple HDV clones from an infected case enabled us to detect such minor strains. The results of this method were confirmed by sequencing of the clones showing a different RFLP pattern. The possibility of contamination was excluded by strict procedures and the analysis of only one case at a time in each experiment. Moreover, sequence analysis of the dominant and minor clones from different individuals also excluded the possibility of contamination. This simple method of RFLP analysis provides a measurement of relative amounts of each component genotype in mixed infections. Moreover, this method enables the investigator to select clones of interest for further virologic studies. The prevalence of mixed infections of HDV may be even higher if more HDV colonies or follow-up serum samples were analyzed for each case.

Consistent with previous studies [Wu et al., 1995b and 1995c], the genotype IIa is not only the most prevalent HDV strain in Taiwanese patients but also the most frequently found genotype in prostitutes. In addition to the three known genotypes, a novel strain of HDV, classified as genotype IIb, was discovered [Wu et al., 1998]. It presented either as the major or the minor strain in cases with mixed genotypes infection. Also, it was found as the only strain in some HDV-infected cases. The micro-heterogeneity in nucleotide sequences of different clones of the dominant HDV strain from a single subject was usually <2%, consistent with the previous report [Wu et al., 1995b]. Although there are arguments as to the evolution rate of the HDV genome [Imazekit et al., 1990; Lee et al., 1992; Netter et al., 1995], divergences of 22.8% to 37% in nucleotide sequences between the newly discovered genotype IIb strain and known genotypes are too great to be explained by evolutionary changes of viral population in an infected case. Therefore, the genotype IIb is not a minor variant or "quasi-species" resulting from evolution. Moreover, the genotype IIb seems to belong to a distinct monophyletic group supported by a bootstrap value of 100% in phylogenetic analysis [Wu et al., 1998]. Taking the genotype IIb into account besides the genotypes I and IIa HDV, mixed genotypes infections of HDV are frequently found in prostitutes who are at high-risk of re-exposure to HDV infection. Although at a lower frequency, mixed HDV genotypes infections have also been found in patients. Furthermore, mixed genotypes infections of HDV were found to secondarily transmit from an infected patient to the spouse. This may have resulted from a single infection of mixed genotypes or from re-infection with another genotype in a patient with pre-existing chronic HDV infection. The impact of mixed infections on the clinical course of HDV infection awaits longitudinal study of more patients.

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